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Ultrastructural Study of an Endogenous Energy Substrate in Spermatozoa of the Sea Urchin *Hemicentrotus pulcherrimus*

MASATOSHI MITA[1,*] AND MASARU NAKAMURA[2]

[1]*Department of Biochemistry, Teikyo University School of Medicine, Itabashi-ku, Tokyo 173, and*
[2]*Department of Zoology, Faculty of Medicine, Teikyo University, Hachioji, Tokyo 192-03, Japan*

Abstract. The morphology of the midpiece in spermatozoa of the sea urchin *Hemicentrotus pulcherrimus* was investigated ultrastructurally with particular emphasis on an endogenous substrate providing energy for motility. The midpiece was composed of a single toroidal mitochondrion surrounding the flagellum. Several lipid bodies (0.1–0.2 μm in diameter) were contained in the space between the mitochondrial outer and inner membranes. Following incubation with seawater, spermatozoa began to swim and the lipid bodies became small and finally disappeared, coincident with a decrease in the level of phosphatidylcholine (PC), an endogenous substrate for energy metabolism. In contrast, during incubation in 100 m*M* K^+-seawater, in which spermatozoa are immotile, there was no decrease in the level of PC and the lipid bodies remained intact. These results strongly suggest that the PC available for use in energy metabolism is located in the lipid bodies within mitochondria in the midpieces of *H. pulcherrimus* spermatozoa.

Introduction

Spermatozoa are stored for months as immotile cells in male sea urchins (Gray, 1928; Rothschild, 1959). Upon spawning in seawater, flagellar movement begins and respiration is activated, in close association with Na^+-dependent acid extraction (Nishioka and Cross, 1978; Christen *et al.*, 1982; Lee *et al.*, 1983; Bibring *et al.*, 1984). Internal alkalization leads to activation of dynein ATPase, resulting in the initiation of motility (Christen *et al.*, 1983).

The energy for flagellar motility of spermatozoa of the sea urchin *Hemicentrotus pulcherrimus* is produced by the oxidation of endogenous phospholipids (Mohri, 1957; Mita and Yasumasu, 1983a). Similar findings have been obtained in many other sea urchins, such as *Echinus esculentus* (Rothschild and Cleland, 1952), *Arbacia lixula* (Mohri, 1964), and *Strongylocentrotus intermedius* (Kozhina *et al.*, 1978). The spermatozoa of *H. pulcherrimus* are generally composed of various phospholipids and cholesterol (Mita and Ueta, 1988, 1989). Triacylglycerol (TG) and glycogen are present in trace amounts (Mita and Yasumasu, 1983a; Mita and Ueta, 1988). The phospholipids include phosphatidylcholine (PC), phosphatidylserine, phosphatidylethanolamine, and cardiolipin. Following incubation with seawater, the level of PC decreases, with no change in the levels of other phospholipids (Mita and Ueta, 1988, 1990; Mita *et al.*, 1990), indicating that PC may be a substrate for energy metabolism in sea urchin spermatozoa. This preferential hydrolysis of PC is related to the properties of phospholipase A_2. The phospholipase A_2 in *H. pulcherrimus* spermatozoa has high substrate specificity for PC (Mita and Ueta, 1990), which may therefore be used specifically for energy metabolism.

Recently, PC has been shown to be abundant in *H. pulcherrimus* sperm midpieces (Mita *et al.*, 1991). Following the initiation of motility, the PC content of sperm midpieces decreases significantly, while that in sperm heads and tails does not change (Mita *et al.*, 1991). Phospholipase A_2 activity is also distributed in the midpieces (Mita *et al.*, 1991). Thus, PC available for use in energy metabolism is located in the midpieces. It has also been reported that the midpieces of *Brissopsis lyrifera* (Afzelius and Mohri, 1966) and *Echinarachinus parma* (Summers and Hylander, 1974) contain a single mitochondrion and lipid globules. The lipid globules are spherical and located in the posterior region between the base of the mitochon-

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* To whom reprint requests should be addressed.

drion and the plasma membrane (Afzelius and Mohri, 1966). Although similar lipid globules have not been observed in spermatozoa of other sea urchin species, it has been reported that lipid bodies are present in *A. punctulata* (Longo and Anderson, 1969) and *A. lixula* (Cosson and Gulik, 1982) spermatozoa. The lipid body differs from the lipid globules, because the former is located inside the mitochondrion and it is relatively smaller than lipid globules (Longo and Anderson, 1969; Cosson and Gulik, 1982). In the present study, the midpieces of *H. pulcherrimus* spermatozoa were examined ultrastructurally to clarify further the energy metabolism of sea urchin spermatozoa.

Materials and Methods

Materials

Spawning of stored spermatozoa of the sea urchin *H. pulcherrimus* was induced by injecting 0.5 *M* KCl into the coelomic cavity. Semen was always collected freshly as "dry sperm" and kept undiluted on ice.

Incubation of spermatozoa

Dry sperm were diluted 100-fold in artificial seawater (ASW) consisting of 458 m*M* NaCl, 9.6 m*M* KCl, 10 m*M* $CaCl_2$, 49 m*M* $MgSO_4$, and 10 m*M* Tris-HCl, pH 8.2. After dilution and incubation at 20°C, the sperm suspension was centrifuged at 3000 × *g* for 5 min at 0°C. In 100 m*M* K^+-seawater, Na^+ was substituted for K^+.

Determination of PC concentration

Total lipids were extracted from spermatozoa using the method of Bligh and Dyer (1959). PC levels were determined by high-performance thin-layer chromatography, as described previously (Macala *et al.*, 1983; Mita and Ueta, 1988). PC content consumed during incubation for 1 h was calculated from the absolute value of PC before and after incubation.

Oxygen consumption

Oxygen consumption in a sperm suspension was measured polarographically with an oxygen consumption recorder (MD-1000, Iijima Electronics MFG Co., Japan). Twenty-five μl of dry sperm were incubated in 2.5 ml of ASW in the closed vessel of the oximeter at 20°C.

Preparation for electron microscopy

Dry sperm were diluted 100-fold in ASW and incubated at 20°C. At appropriate intervals, the spermatozoa were prefixed in 2.5% glutaraldehyde ASW solution for 40–60 min at 4°C; a volume of sperm suspension was mixed with the same volume of cold 5% glutaraldehyde in 80% ASW. The prefixed spermatozoa were rinsed with cold ASW and post-fixed with 1% OsO_4 in ASW for 2 h at 4°C. Samples were washed in distilled water, and then immersed in saturated aqueous uranyl acetate for 1 h for block staining. After dehydration in a graded series of ethanol solutions, the specimens were embedded in epoxy resin and ultrathin sections were cut on a Reichert Ultracut ultramicrotome. After staining the specimens with lead citrate, we used a Hitachi 7000 or JEM 100 CX electron microscope to observe them.

Results

In longitudinal sections through spermatozoa of *H. pulcherrimus*, the midpiece was observed to consist of a single toroidal mitochondrion (Fig. 1). The midpiece did not contain the lipid globules observed in the spermatozoa of *B. lyrifera* (Afzelius and Mohri, 1966) and *E. parma* (Summers and Hylander, 1974). A region between the mitochondrial outer and inner membranes—intramembrane space—was dilated in a band nearest the flagellum

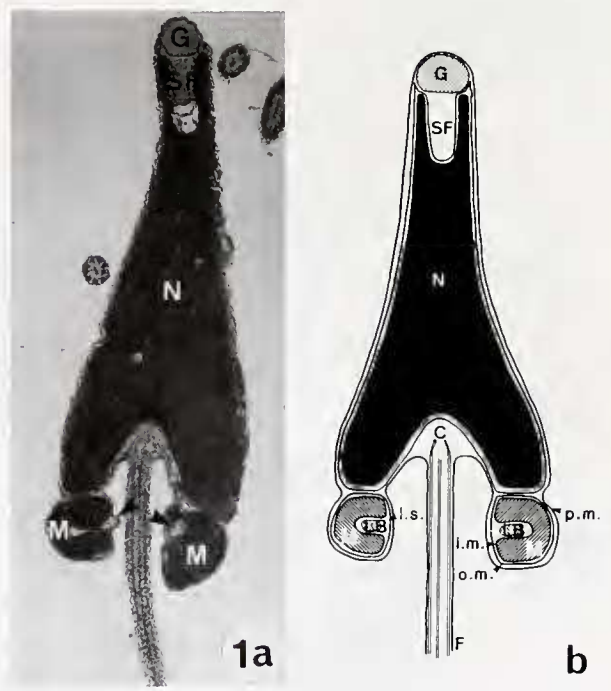

Figure 1. Longitudinal section (a) and schematic representation (b) of a spermatozoon of *Hemicentrotus pulcherrimus*. Arrow heads show lipid bodies (LB). C: proximal centriole, F: flagellum, G: acrosomal granule, M: mitochondrion, N: nucleus, o.m.: mitochondrial outer membrane, p.m.: plasma membrane, SF: subacrosomal fossa, i.s.: intramembrane space. ×19,700.

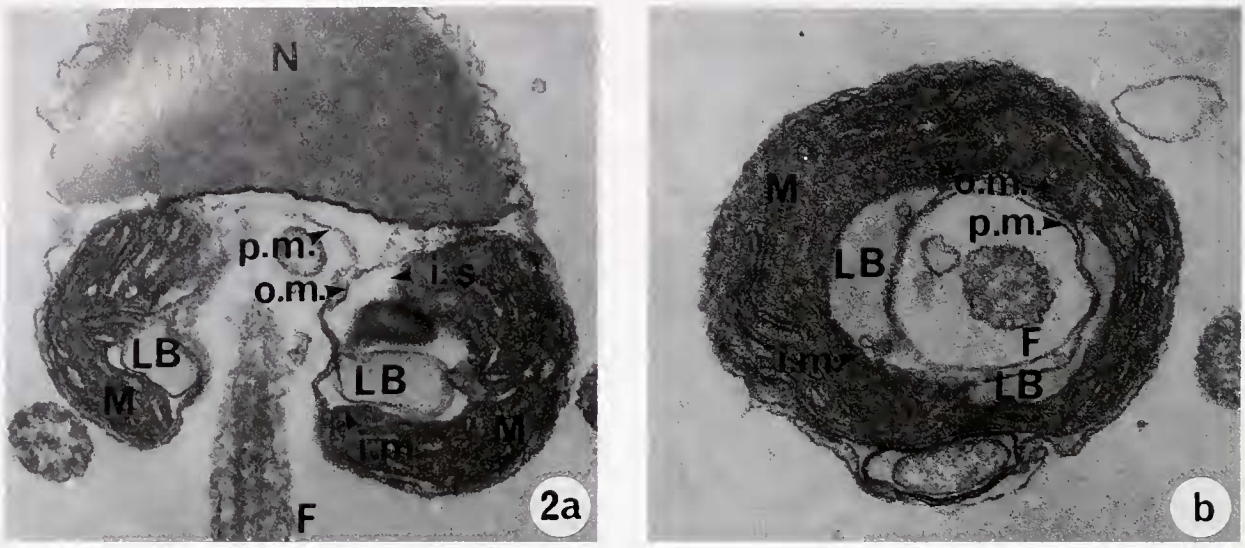

Figure 2. Longitudinal (a) and transverse (b) sections through the mitochondrial region of spermatozoa before incubation in seawater. F: flagellum, i.m.: inner mitochondrial membrane, i.s.: intramembrane space, LB: lipid body, M: mitochondrion, N: nucleus, o.m.: outer mitochondrial membrane, p.m.: plasma membrane. ×58,800.

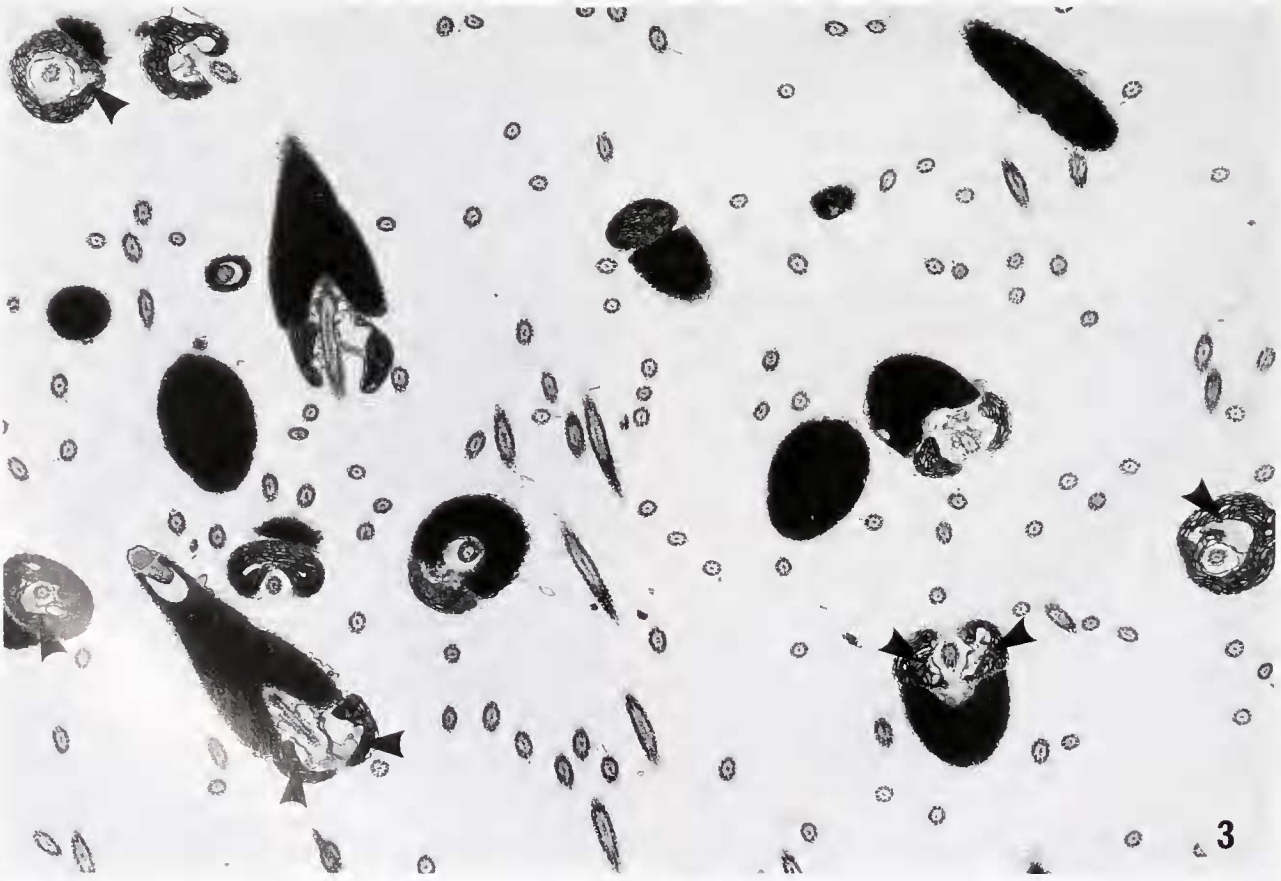

Figure 3. Electron micrograph of spermatozoa before incubation with seawater. Arrow heads show lipid bodies. ×11,800.

and contained low-electron-density lipid bodies (Fig. 2). These lipid bodies were irregular in profile and about 0.1–0.2 μm in diameter. All of the spermatozoa in semen contained the lipid bodies within their mitochondria (Fig. 3). The same lipid bodies were also observed in spermatozoa present in the testis (data not shown).

When dry sperm were diluted and incubated in ASW, spermatozoa began to swim and the amount of sperm PC decreased (Fig. 4). About 6 μg of PC was consumed in 10^9 spermatozoa following incubation for 1 h (Table I). In addition to PC consumption, respiration was activated and about 0.27 μmol O_2/h/10^9 spermatozoa was consumed. These findings confirm the previous observations (Mita and Ueta, 1988, 1990). Longitudinal and transverse sections of the midpieces of spermatozoa were examined following incubation in ASW. After 5 min of incubation, changes were noted in the structure of the inner ring of the mitochondrion. Although lipid bodies were still present, they had shrunk. In addition, a gap was observed to have opened between the plasma membrane and the mitochondrial outer membrane (Fig. 5b, e). After 30 min of incubation, the inclusion bodies and the inner ring of the mitochondrion had disappeared (Fig. 5c, f). Various structural features of the mitochondrion, such as the number of cristae and the thickness of the membranes, did not change during incubation in ASW.

Because sea urchin spermatozoa incubated in high K^+-seawater are immotile and their respiration extremely low (Schackmann *et al.*, 1981; Mita and Yasumasu, 1983b, 1984), the effect of a high-K^+ environment on the lipid bodies of the midpiece was examined. After incubation in 100 m*M* K^+-seawater for 1 h at 20°C, neither oxygen nor PC was consumed by the spermatozoa (Table I), and the lipid bodies of the midpiece remained intact (Fig. 6a, b). Thus, the disappearance of the lipid bodies was correlated with the decrease in the level of PC.

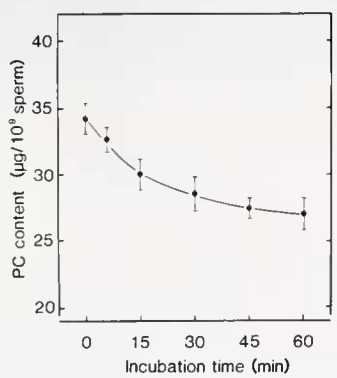

Figure 4. The change in level of phosphatidylcholine (PC) in sea urchin spermatozoa following incubation in seawater. Each value is the mean of four separate experiments. Vertical bars show S.E.M.

Table I

Phosphatidylcholine and oxygen consumption in sea urchin spermatozoa

Conditions	PC consumption (μg/h/10^9 sperm)	O_2 consumption (μmol O_2/h/10^9 sperm)
Seawater	6 ± 1	0.27 ± 0.02
100 m*M* K^+-seawater	N.D.	<0.01

Dry sperm were diluted 100-fold in either seawater or 100 m*M* K^+-seawater and incubated for 1 h at 20°C. Values are means ± S.E.M. obtained from four separate experiments. N.D., not detectable.

Discussion

The present study demonstrated lipid bodies in the intramembrane space of the mitochondrion in the sperm midpiece of *H. pulcherrimus* (Fig. 1). Following incubation of spermatozoa in seawater, these lipid bodies disappeared gradually (Fig. 5), although they still remained after incubation in 100 m*M* K^+-seawater (Fig. 6). These observations were correlated with changes in the level of intracellular PC (Fig. 4), suggesting that PC available for use in energy metabolism is related to the lipid bodies within the mitochondria of the midpiece. Similar lipid bodies have been observed in the spermatozoa of *A. punctulata* (Longo and Anderson, 1969) and *A. lixula* (Cossin and Glik, 1982). It has also been reported that *A. lixula* spermatozoa obtain energy for movement from the oxidation of endogenous phospholipid (Mohri, 1964). These findings also support the hypothesis that the lipid bodies within mitochondria are reservoirs of endogenous PC substrate in sea urchin spermatozoa.

We also showed that about 6 μg of PC was consumed in 10^9 spermatozoa following incubation for 1 h (Fig. 4, Table I). Because this amount was only $^1/_5$ of the total PC, the remaining $^4/_5$ of cellular PC may be membrane-bound and therefore inaccessible as an energy substrate for motility. About 0.27 μmol O_2/h/10^9 spermatozoa were consumed (Table I). This degree of oxygen consumption is enough to account for the consumed PC, as mentioned previously (Mita and Yasumasu, 1983; Mita *et al.*, 1990). Presumably, the fatty acid liberated from PC in the lipid bodies is metabolized through β-oxidation to produce ATP.

Unfortunately, there is little direct evidence to indicate whether the content of the lipid bodies is, in fact, PC. A cytochemical study would be useful to identify PC in the lipid bodies, although an antibody against PC would be difficult to prepare because PC is a common membrane component. We are now investigating the role and characteristics of the lipid bodies to provide useful insights

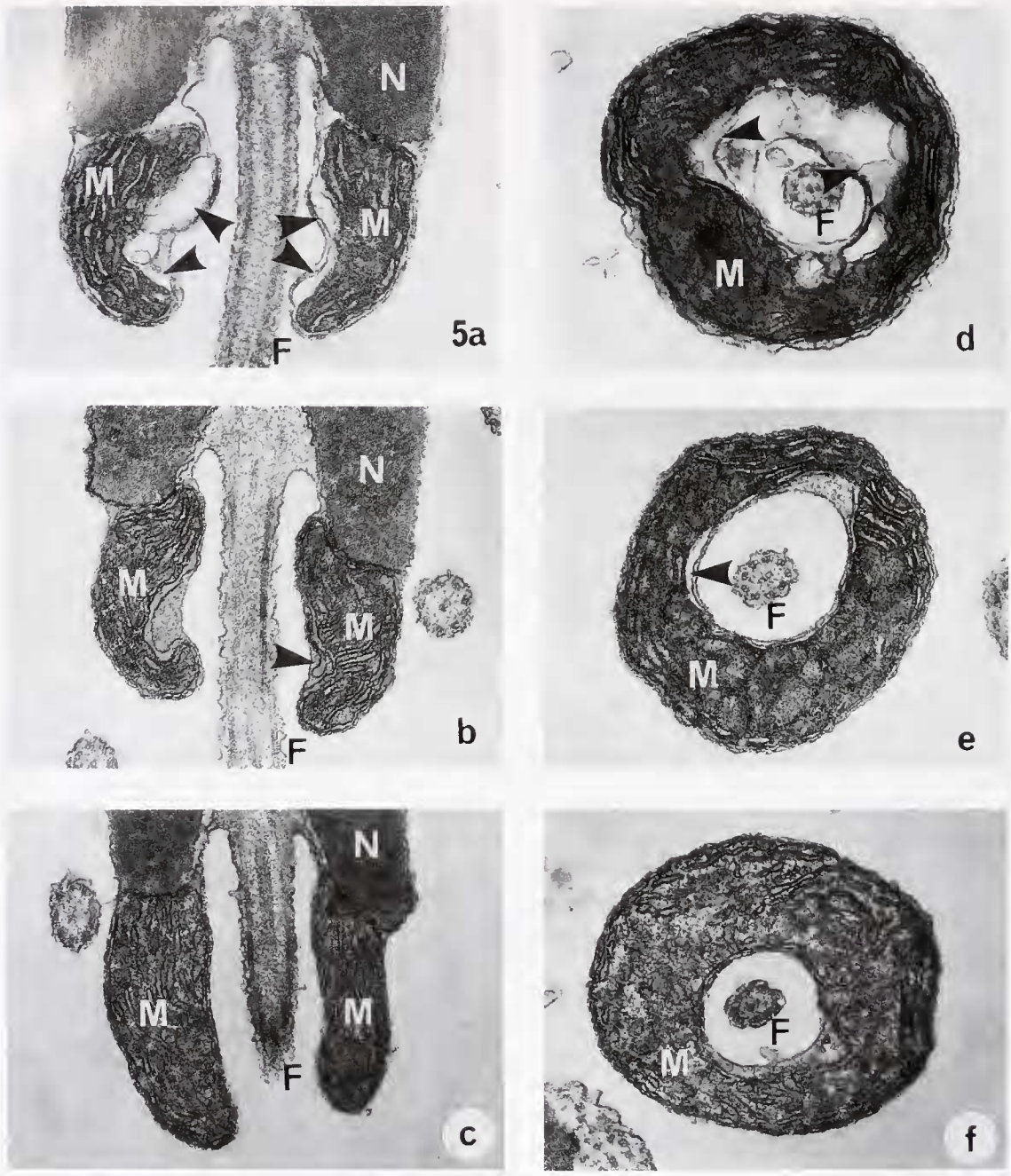

Figure 5. Longitudinal (a–c) and transverse (d–f) sections through the mitochondrial region of spermatozoa before (a, d) and after incubation in seawater for 5 min (b, e) and 30 min (c, f). Arrow heads show lipid bodies. F: flagellum, M: mitochondrion, N: nucleus. ×42,500.

into the direct mechanism of energy metabolism in sea urchin spermatozoa.

In contrast to the PC used in *H. pulcherrimus*, *Glyptocidaris crenularis* spermatozoa use TG as a substrate for energy metabolism (Mita, 1991). There are several lipid globules at the bottom of the midpiece in *G. crenularis* spermatozoa (Mita and Nakamura, 1992), similar to those in the spermatozoa of *B. lyrifera* (Afzelius and Mohri, 1966) and *E. parma* (Summers and Hylander, 1974). After incubating *G. crenularis* spermatozoa with seawater, both the number and the size of the lipid globules decreased, coincident with a decrease in the TG level.

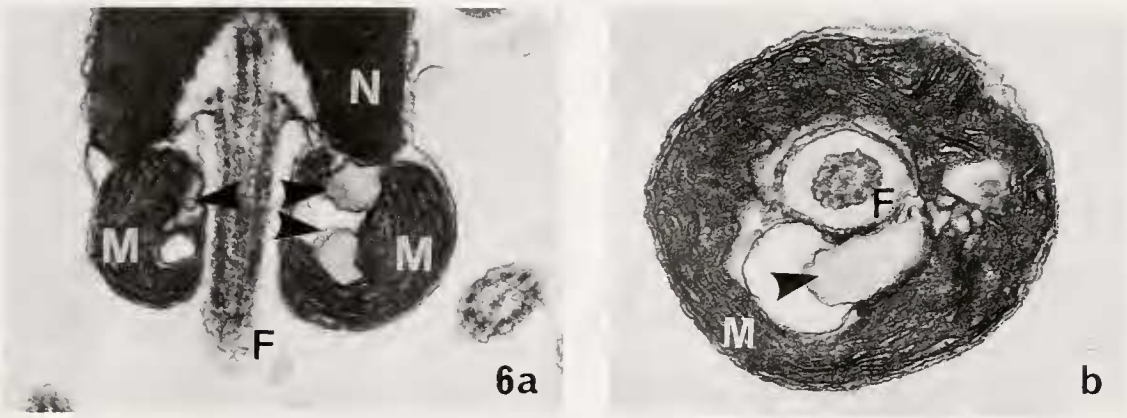

Figure 6. Longitudinal (a) and transverse (b) sections through the mitochondrial region of spermatozoa after incubation in 100 m*M* K^+-seawater for 1 h. Arrow heads show lipid bodies. F: flagellum, M: mitochondrion, N: nucleus. ×42,500.

However, neither TG (Mita and Ueta, 1988) nor lipid globules (Fig. 1) are present in *H. pulcherrimus* spermatozoa. Thus it appears that TG is related to the lipid globules.

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